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The Effect of Spore Density on Germination and Development in *Pteridium*, Monitored using a Novel Culture Technique

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ABSTRACT.—Percentage germination and percentage transition to two-dimensional growth in *Pter-idium* (bracken fern) were recorded for spores sown on a solid Phytagel[®]-based growth medium at known densities of between 3 and 9883 spores mm⁻². The maximum germination recorded occurred at sowing densities higher than those at which maximum transition occurred. Percentage germination was greatest (43 to 52% of spores sown) at intermediate densities (187 to 2114 spores mm⁻²), being highest (52% of spores sown) at 360 spores mm⁻². Percentage transition was highest at the lowest densities used. It was concluded that germination and transition have different density optima and that investigations of these two phenomena in ferns should take account of this.

Fern gametophytes are accepted as excellent models for the study of many biological systems. Their utility in the studies of both pure and applied developmental biology and genetics was described by Hickok *et al.* (1987), see also Miller (1968). Their usefulness for the study of developmental selection was illustrated by Klekowski (e.g. 1982). Fern gametophytes have also been used in studies of herbicide tolerance and herbicide mode of action, (Hickok *et al.*, 1987; Keary *et al.*, 2000). However, the density of fern spores on their substrate affects both percentage germination and gametophyte development (see Dyer, 1979 for review). The findings reviewed by Dyer which related to the sowing of spores at known densities on artificial media indicated that percentage germination is inhibited at both high and low densities, yet few fern researchers before or since have specified the spore densities used in their experiments. It follows that fern gametophyte studies would benefit from being conducted at optimal densities for the phenomenon under study; there is a need for quantification.

Two problems arise in attempting to quantify the effects of spore sowing density on germination and transition. Transition is a term used to describe the progression from (1D) filamentous growth to (2D) growth involving divisions in more than one plane, which generates the thallus in most fern gametophytes. The first problem is obtaining an even distribution of spores across the culture medium. Carboxy-methyl cellulose (CMC, manufactured by BDH) has been used to help spread spores evenly over media surfaces (e.g.

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Sheffield *et al.*, 1997). CMC does aid spore dispersal in liquid but does not ensure even distribution across the entire surface of solidified growth media. Even spore distribution can be achieved by spraying using a diffuser (see Dyer, 1979). However, this method only works well on a large scale and is wasteful, an important consideration in work with rare or irregularly fertile species. Given the toxicity of fern spores (Povey *et al.*, 1995; Simán *et al.* 1999; Simán *et al.* 2000), the generation of aerosols containing spores raises health and safety considerations. A second problem concerns counting spores and scoring germination and transition in cultures sown at high densities. At high densities, spores and gametophytes cover one another and make germination difficult to detect.

The aim of this work was to quantify the effect of spore sowing density on germination and transition to two-dimensional growth in *Pteridium*. A new culture technique was developed to allow this.

METHODS

Two experiments were conducted under identical conditions, unless otherwise stated: Experiment 1 and Experiment 2. Both experiments used spores of Pteridium aquilinum collected in 1990 from northern Majorca, Balearic Islands (Spain). These spores are E.S. Collection Number 196. At the time of collection, rhizome material was also taken. This was planted at the University of Manchester Botanical Grounds where the specimen now forms part of the living collection. Spores were stored in sealed plastic vials at 4 °C after collection and until surface sterilisation in 1999. Moore's medium (after Moore, 1903), pH 6.5, was used as the growth medium, with the addition of Phytagel[®] (manufactured by Sigma) gelling agent (0.2 % weight/volume). Phytagel[®] is a gelling agent that forms a solid similar in form to that of conventional agar media. Phytagel[®] is distinct from traditional gelling agents in that when agitated, it changes phase from a solid to a liquid. The Phytagel[®]-based growth medium was autoclaved then allowed to cool to approximately 40°C. In the first experiment (Experiment 1), 100 µl of Phytagel[®]-based medium was dispensed into each 2.35 mm diameter well microtitre plate (approximate well volume 175 µl). A microtitre plate is a transparent plastic tray, with 96 individual wells). In the second experiment (Experiment 2), 200 µl of Phytagel[®]-based medium was dispensed into each 6.65 mm diameter well microtitre plate (approximate well volume 370 µl). Microtitre plates were left open until the surface of the medium was dry. Lids were then sealed in place with Parafilm (manufactured by NESCO). All equipment and solutions were autoclaved or purchased sterile. Procedures were conducted in an Envar laminar flow cabinet at room temperature. Spores were prepared by soaking overnight at room temperature in 15 ml of dH2O, to which had been added 2 drops of Tween 80 detergent (BDH). Tween 80 acts as a wetting agent and serves to disaggregate clumps of spores. Spore suspensions were then poured into a Sartorius vacuum filter unit into which had been fitted a 0.45µm nitro-cellulose membrane (manufactured by What-

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TABLE 1. The spore sowing densities used (spores mm⁻² of medium surface).

Treatment	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13
Experiment	1	1	2	2	1	2	2	2	2	2	1	2	2
Density	3	35	187	360	538	618	1137	2414	4445	5671	7813	7859	9883

man). The Tween 80 solution was then filtered off from the spores. The spores were then surface sterilised by re-suspending in 50 ml of 5% (vol/vol) aq. sodium hypochlorite (approximately 10-14% available chlorine, BDH) for three minutes. The sodium hypochlorite solution was filtered off and the spores rinsed three times each with 50 ml of sterile dH₂O. In order to facilitate the inoculation of the growth medium, surface sterilised spores were suspended in a solution of 0.5% (w/vol) CMC (BDH) in sterile dH₂O. This was done by removing the nitro-cellulose membrane from the filter unit and eluting the spores from the membrane using a 1 ml Gilson pipette. In the case of the highest density treatments, inoculation was conducted using a fixed volume of undiluted stock spore suspension. In the case of other density treatments, aliquots were taken from this stock spore suspension and made up to the same fixed volume of inoculum that was used to inoculate at the highest densities. The 2.35 mm diameter microtitre plate wells used in Experiment 1 were inoculated with 10 µl of inoculum. The 6.65 mm diameter microtitre plate wells used in Experiment 2 were inoculated with 20 µl of inoculum. The spore suspension flowed across the surface of the growth medium, distributing the spores evenly. In Experiment 1, spores were sown at four densities (Table 1). In Experiment 2, spores were sown at nine densities (Table 1). A total of thirteen densities were sown, d1 to d13. For each experiment, four replicate blocks were sown on the same microtitre plate. The experiment was designed so that there was an overlap in the ranges of spores sown in the two experiments. To illustrate the repeatability of the culture technique, one spore density was repeated in both experiments; d11 from Experiment 1 was sown at approximately the same density as d12 from Experiment 2 (see Table 2).

Spores were grown in sealed microtitre plates maintained at 20 \pm 2 °C with 12 h light, 12 h dark cycles of illumination for 14 days. Approximate photon flux density was 120 μ mol.m⁻²s⁻¹.

At the end of the growth period spores were re-suspended using gradual additions of known volumes of 0.5% (w/vol) CMC (BDH) in sterile dH₂O to

each treatment until all spores were re-suspended. Gentle fluxing back and forth using a 1 ml Gilson pipette caused the Phytagel[®] to change phase from a solid to a liquid, thereby re-suspending the spores and gametophytes that had previously been evenly spread across surface of the solid medium. Each final spore suspension was rendered sufficiently dilute to enable convenient scoring using a Sedgwick Rafter counting chamber and viewed under a Leitz Dialux 20EB binocular compound microscope at ×400 magnification. Final volumes of spore suspension (including the volume of Phytagel[®]-based

TABLE 2. Mann-Whitney U test of d11 versus d12 for density, germination and transition.

	U-Value	Tied Z-Value	Tied P-Value	
Density	51222	0.079	0.9369	
Germination	51120	1.271	0.2037	
Transition	48861	1.086	0.2775	

growth medium) ranged from the equivalent of 0.2 ml to 32.4 ml, depending on spore density.

Following re-suspension, sampling was such that two or three 1 ml aliquots were taken from each treatment final spore suspension, with the exception of treatments with final suspension volumes of less than 2 ml. In such cases, the whole final spore suspension was sampled. Between 266 and 431 one-microlitre counting chamber cells were scored for each treatment sowing density. A total of 4386 one-microlitre counting chamber cells were examined and 7228 spores or gametophytes were recorded. Germination was considered to have occurred with the emergence of a rhizoid.

RESULTS

Preliminary analysis of both experiments, using Mann-Whitney U-tests, showed there to be no difference between replicate treatments (p < 0.01) and also that there was no significant difference between replicate 1 ml aliquots taken from a given treatment spore suspension (p < 0.01). Consequently, values recorded from each counting chamber cell were treated as individual estimates of spore density, germination and transition. Means and confidence intervals were calculated using these estimates. The data were not normally distributed. Accordingly, spore density estimates were log-transformed and percentage germination and percentage transition data were arc-sin transformed. Ninety five percent confidence intervals were calculated using transformed data according to Wheater & Cook (1999). All statistics were conducted using StatView and graphs produced using Excel. The spore densities tested are shown in Figure 1 and listed in Table 1. Percentage germination was greatest (43 to 52% of spores sown) at intermediate densities, 187 to 2114 spores mm⁻² (d3 to d8), being highest (52% of spores sown) at 360 spores mm⁻² (d4). Percentage transition was greatest (between 0.5 and 1.4% of spores sown) at lower densities (3 to 538 spores mm⁻²). At densities of 4445 spores mm⁻² and above, percentage transition did not exceed 0.00005%. Percentage transition was zero at densities of 7813 and 9883 spores mm⁻². Far greater variance was observed for transition than for germination. The two treatments sown at the same density in different experiments, d11 and d12, showed the same proportions of germination and transition, see Table 2.



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FIG. 1. Sowing density (a), percentage germination (b) and percentage transition (c) for *Pteridium* spores at 13 densities (d1-d13). Open symbols + Experiment 1, closed symbols = Experiment 2. Error bars = 95% confidence intervals.

DISCUSSION

The period of time for which Pteridium spores remain viable varies. Pteridium spores do not remain viable for long in soil under field conditions (Conway, 1953; Lindsay et al., 1994). In contrast, it seems that spores stored in sealed containers at 4°C may remain viable for many years (Simpson & Dyer, 1999). Our findings show that germination percentage as high as 52% may occur in spores stored in this way for 9 years, this is higher than the percentage germination obtained from 9 year old spores in the study of Lindsay et al. (1994). It should be noted that subsequently, transition was low, less than 1.5% in all cases, but the reasons for this were not sought or identified. Sheffield (1996) reported that Pteridium spores collected from the same location in different years can differ considerably in percentage germination. It may be that the environmental conditions to which a frond is exposed have an effect on the viability of spores produced by that frond. One environmental variable that is known to influence germination is temperature. Pangua et al. (1994), for example, presented evidence temperature influences germination in three species of Asplenium. Percentage germination of Pteridium spores collected in the same year from different localities also varies, as does germination in spores stored for different lengths of time, but there is no direct relationship between Pteridium spore age and viability (Lindsay et al., 1994; Sheffield, 1996). Given effects of hydration (Lindsay, Williams and Dyer, 1992), growing temperature and storage time on percentage germination, it is clear that storage conditions have an important influence on fern spore percentage germination. It would be valuable if standardised spore conditions could be agreed, or at least that futureauthors were encouraged to include a statement describing spore storage conditions. Although germination of many species might be higher following fully hydrated storage (Lindsay et al., 1992) this method is space and time consuming and inappropriate for species, such as Pteridium, capable of dark germination. We therefore suggest that pteridophytes spores should routinely be stored dry, in sealed containers at 4°C, in the dark. Not only are spores inherently variable in terms of germination, but our experiments show that they are also sensitive to growing conditions. The density at which Pteridium spores are sown on their substrate affects germination and development. Percentage germination was highest at densities intermediate in the range tested herein. This is in keeping with earlier findings, such as those reviewed by Dyer (1979). Smith & Rogan (1970) found an inhibition of development by gametophytes of Polypodium vulgare to increase with spore sowing density. Smith & Robinson (1971) identified three growth factors responsible for the inhibition of cell division in high densities of Polypodium vulgare. They concluded that other factors, such as competition for nutrients and low light intensity through mutual shading, may be involved, but were probably important at later stages of development and at extremely high densities. Light is not required for germination of Pteridium spores (Lindsay et al., 1994) but is required for photosynthesis to drive gametophyte development.

Smith & Robinson's (1971) investigations into the effect of high spore density were done in liquid growth media. We have expressed our densities on the basis of numbers on the surface of the medium, and undoubtedly depth of medium and speed of diffusion of nutrients into, and exudates from, gametophytes influences results in different vessels or types of solidified medium. We attribute impeded development of Pteridium at extremely high sowing densities to both resource limitations and inhibition due to gametophyte exudates. As for storage conditions, discussed above, it is clear that density does have an important influence on germination and growth, and we recommend that future authors standardise, or at least state, their spore sowing densities. There are ecological implications to density-dependent germination rates. The majority of fern spores probably fall within a few metres of their sporangia but a few travel great distances, see Sheffield (1996) and Simán et al. (1999). Our results imply that spores landing close to the parental sporophyte could accumulate to such a high density that germination and gametophyte development are inhibited; the reverse would apply to spores dispersed over great distances. Ecological implications are that lone or sparse spores are unlikely to germinate but if they do, they are likely to undergo transition and therefore go on to produce female gametophytes. This will mean that these female gametophytes will secrete antheridiogens and promote germination in any other spores in the area, and cause those neighbouring spores germinate develop into male gametophytes (Näf, 1958).

The new culture technique employed in this work provides an economical and straightforward means of determining spore density and quantifying germination and transition. The repeatability of the technique was illustrated by the identical germination and transition percentages observed in d11 from Experiment 1 and d12 from Experiment 2, two treatments sown at identical densities in separate experiments. Laboratory conditions were optimised during the course of preliminary work. In particular, the volume of inoculum used is critical. The volumes presented here were optimised in a laminar flow cabinet at room temperature and are intended only for guidance. Other laboratories may have slightly different environmental conditions, e.g. room temperature, and it may be necessary to fine-tune procedures for local conditions. Preliminary trial and error optimisation is recommended. It was observed during our preliminary experiments that sub-optimal conditions lead to shrinkage of inoculum upon drying, resulting in a distribution of spores which did not extend to the full circumference of the microtitre plate well. In such situations it may still be possible to quantify spore density by estimating the diameter of dried spore inoculum and re-calculating accordingly, using this diameter, rather than that of the microtitre plate well. In conclusion, Pteridium spores stored for nine years at 4°C and sown at 360 spores mm⁻² had a mean maximum germination rate of 52%. Lower mean maximum germination rates were observed above and below this density. Inhibition of germination at high densities may be due to resource limitation, physical impediment or biochemical inhibition caused by the exudation of

growth inhibitors. Inhibition of germination at low densities may have a biochemical basis; germination may be stimulated by the exudation of growth promoters from other spores. At higher spore densities, the effects of growth inhibitors, physical impediment or resource limitation overcome the effects of growth promoters. The low percentage transition observed could be a consequence of the age of the spores used. Inhibition of transition at high densities may be due to resource limitation, but further work is required to test these last two hypotheses.

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